REMARKS

Claims 1 and 3 are pending after entry of this paper. Claims 1 and 3 have been rejected. Claims 2 and 4 have been cancelled without prejudice. Applicants reserve the right to pursue cancelled claims in a continuing application.

Claims 1 and 3 have been amended to a method of treating or preventing pemphigus by administering an effective amount of a composition containing an anti-CD40L antibody, as an active ingredient. Support may be found throughout the instant specification, for example, at page 5, first paragraph.

No new matter has been introduced by these amendments. Reconsideration and withdrawal of the pending rejections in view of the above claim amendments and below remarks are respectfully requested.

Response to Rejections under 35 U.S.C. §112

Claim 3 stands rejected under 35 U.S.C. §112, first paragraph for lack of enablement. Specifically, the Examiner contends that the claimed invention allegedly does not provide direction on how to make and use the information that certain people were predisposed to pemphigus or to utilize anti-CD40L antibodies to prevent the recurrence of the disease (Office Action – page 3). The Examiner further alleges that the applicant has not sufficiently addressed the unpredictability and inconsistency of treating patients with pemphigus. In particular, the Examiner states that "it is not clear that reliance on the *in vitro* and *in vivo* experimental observations as well as the clinical experience with targeting various inflammatory conditions with CD40L-specific antibodies accurately reflects the relative ability of efficacy of the claimed

'preventive agents' to prevent pemphigus" (Office Action – page 3). Accordingly, the Examiner concludes that in view of the lack of predictability of the art and the lack of established clinical protocols for effective methods to prevent pemphigus, undue experimentation would be required (Office Action – page 5). Applicants respectfully disagree.

Applicants assert that contrary to the Examiner's contention, one skilled in the art could make and use the claimed invention without undue experimentation. In general, preventive medicine is attributed to preventing certain diseases prior to their onset or recurrence. It is well recognized in the art that certain traits, genetic or otherwise, can influence an individual's predisposition for certain diseases.

In the case of pemphigus, it has been conclusively shown in the previous response dated June 29, 2007, that certain people are predisposed to the pemphigus disorder. For example, Starzycki, et al. (*International Journal of Dermatology*. 37(3):211-214, March 1998), Katzenelson, et al. (*Dermatologica*, 1990;181(1):48-50) and Tur, et al. (*Arch Dermatol.* 1998 Nov;134(11):1406-10) (copies of abstracts previously submitted). Therefore, these individuals could benefit from preventive measures against the onset of the phemphigus disorder.

To demonstrate the preventive capabilities of the claimed invention, Example 2 demonstrates a treatment where autoantigen-knockout mice, recognized in the art to represent an active autoimmune disease model for pemphigus (Amagai, et al. *The Journal of Clinical Investigation* 105(5):625-631, 2000; respectfully submitted), were succesfully administered with a composition having anti-CD40L antibody prior to the onset of pemphigus to prevent the symptoms of pemphigus from occuring. Similarly, a patient who has a high likelihood of developing pemphigus, for example in Katzenelson, et al. a "woman and her son developed the disease within a period of 18 months from one another,", would be a potential candidate in the

preventive immunosuppressant therapy using the claimed method, where the disease would be inhibited from developing in the yet symptoms-free person. Thus, one skilled in the art could make and use the information that certain people are predisposed to pemphigus to utilize anti-CD40L antibodies to prevent the onset or recurrence of the disease.

Moreover, in the last paragraph on page 3 of the Office Action, the Examiner questions the use of *in vitro* and animal model studies to establish the relative ability and efficacy of the claimed preventive utility of anti-CD40L antibody. However, there are numerous publications available where mouse models have been utilized to study the preventive therapies, for example, Park, et al. "Preventive effect of antioxidants in MPTP-induced mouse model of Parkinson's disease" (*Neuroscience Letters*, 363(3):243-246, 2004; abstract respectfully submitted). The applicant clearly establishes cause and effect between the pathogenicity of IgG autoantibodies against desmoglein 3 (Dsg3) and the onset of the pemphigus disorder in the specification as originally filed, which is further supported by the inventors publication (Amagai, et al. *The Journal of Clinical Investigation* 105(5):625-631, 2000) incorporated by reference. Thus, a mouse model that can mimic the production of anti-Dsg3 IgG autoantibodies can be employed in the study of pemphigus (Amagai, et al.). For more details on how the mouse model was developed the applicant wishes to direct the Examiner's attention to the Results section of Amagai, et al. Since it was established that the onset of the pathogenicity of IgG autoantibodies against Dsg3 and resultant phenotypes of pemphigus disorder occur upon recognition of Dsg3 antigen by anti-Dsg3 IgG autoantibodies, an agent that can be administered prior to the onset of the pemphigus disorder that would inhibit the pathogenicity of IgG autoantibodies against Dsg3 can be used in a preventive therapy. Example 2 demonstrates that anti-CD40L antibodies (MR1) administered to the Rag2(-/-) mouse, which expresses Dsg3, prior to the introduction of Dsg3(-/-

) splenocytes prevented the production of anti-Dsg3 antibodies or pemphigus phenotype after the introduction of Dsg3(-/-) splenocytes, whereas the control group of Rag2(-/-) mice with immunized Dsg(-/-) splenocytes without previously introduced anti-CD40L antibodies (MR1) has shown all the symptoms of the pemphigus disorder. In this instance, one skilled in the art readily understands that the introduction of anti-CD40L antibodies prior to the onset of symptoms of pemphigus as observed in the control group was preventive, and the use of the animal model to establish relative ability and efficacy of the claimed preventive utility of anti-CD40L antibody would be also readily recognized.

Furthermore, applicant once more wishes to assert in reference to the Office Action at page 4, that the MPEP guidelines as presented in MPEP §2107.03 (III) state that "[d]ata from *in vitro* or animal testing is generally sufficient to support therapeutic utility." Additionally, MPEP §2107.03 (V) states that "[o]ffice personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials." Thus, according to the MPEP guidelines the therapeutic utility demonstrated in the animal testing provided by the applicants, *i.e.*, use of the autoantigen-knockout mouse model system, is sufficient for demonstrating enablement.

Moreover, the Examiner quotes from *The Merck Manual of Diagnosis and Therapy* (the Merck Manual) that "Pemphigus is a serious disease with an inconsistent and unpredictable response to therapy and that the aim of treatment is to stop the eruption of new lesions" to establish that the treatment should only occur after an individual being diagnosed with pemphigus (Office Action – page 4). However, applicants wish to direct the Examiner's attention to the fact that pemphigus is normally treated with non-specific immunosuppressant agents, such corticosteroids or milder oral steroids, especially prednisone (page 829 of the Merck

Manual). However, these drugs may cause severe side effects ("virtually inevitable complicating drug side effects" see page 830 of the Merck Manual) and thus require such stringent guidelines as presented by the Examiner. However, the claimed invention involves a highly specific immunosuppressant therapy that can potentially be utilized as a preventive measure in the individual predisposed to pemphigus disorder. Thus, one skilled in the art would not apply the same guidelines as recited in *The Merck Manual of Diagnosis and Therapy* intended for the use with a non-specific immunosuppressant agents to a highly specific immunosuppressant therapy based on administering anti-CD40L antibodies of the present invention.

For the reasons stated above, at the time of the invention, one skilled in the art would have considered having read the instant specification, that the claimed method of preventing pemphigus does not necessitate undue experimentation to use and/or make the same, and would be enabled to practice the claimed invention. Reconsideration and withdrawal of the enablement rejection under 35 U.S.C. §112, first paragraph are respectfully requested.

Response to Rejections under 35 U.S.C. §102

Claims 1 and 3 have been rejected under 35 U.S.C. §102(b) as being anticipated by U.S. Patent No. 6,001,358 to Black, et al. Specifically, according to the Examiner, Black allegedly discloses anti-CD40L antibody and therapeutic compositions thereof (Office Action – page 6). Accordingly, the Examiner alleges that the claimed invention is anticipated by such dislcosure. Applicants respectfully disagree.

However, in order to expedite prosecution and without disclaimer of or prejudice to the subject matter recited therein, applicants have amended claims 1 and 3 to recite the

claimed invention as a method of treating (claim 1) or preventing (claim 3) pemphigus by administering a pharmaceutically effective amount of a composition comprising an anti-CD40L antibody.

As noted in the previous response dated June 29, 2007, Black characterizes humanized antibodies to human gp39 (also called CD40L; Column 3, line 46 of Black). In particular, Black discloses that an anti-CD40L antibody can be potentially used to treat 141 autoimmine and non-autoimmune conditions (paragraph bridging columns 32 and 33).

As the Examiner is aware:

> [a] reference itself must have an enabling disclosure to be used as a proper reference. Section 102(b) of 35 U.S.C. and its predecessor statues have been interpreted as requiring the description of the invention in a publication to be sufficient to put the public in possession of the invention. *Ex parte Gould*, 231 U.S.P.Q. 943 (B.P.A.I. 1986).

In fact, Black recites 18 preferred indications treatable by administration of anti-CD40L antibodies, but does not include pemphigus (Column 33, lines 23-31). Merely listing all possible immune related diseases does not provide an enabling disclosure to one skilled in the art to practice the claimed invention without an undue experimentation. In fact, one skilled in the art would have to perform a great deal of undue experimentation to arrive at the claimed invention.

Therefore, the applicant asserts that a non-specific disclosure with respect to a potential use of antagonistic anti-CD40L antibodies for the treatment of autoimmune and non-autoimmune conditions (total of 141 conditions listed by Black) including pemphigus (Office Action – page 6) cannot be anticipatory of the claimed method of treating and preventing pemphigus. As previously noted, simply because a moiety is listed as one possible choice for

one position does not mean there is *ipsis verbis* support for every species or subgenus that chooses that moiety. Were this the case, a "laundry list" disclosure of every possible moiety for every possible position would constitute a written description of every species in the genus. *Fujikawa v. Watanasin*, 93 F.3d 1559, 39 U.S.P.Q.2d 1895, 1905 (Fed. Cir. 1996).

Therefore, applicants assert that Black does not anticipate the claimed invention because Black does not disclose a method for treating or preventing pemphigus by administering a composition containing anti-CD40L antibody as presently claimed. Reconsideration and withdrawal of the §102(b) rejection to claims 1-4 are respectfully requested.

Thus, applicants respectfully submit that the invention as recited in the claims as presented herein is allowable over the art of record, and respectfully request that the respective rejections be withdrawn.

CONCLUSION

Based on the foregoing amendments and remarks, the applicants respectfully request reconsideration and withdrawal of the pending rejections and allowance of this application. The applicants respectfully submit that the instant application is in condition for allowance. Entry of the amendment and an action passing this case to issue is therefore respectfully requested. In the event that a telephone conference would facilitate examination of this application in any way, the Examiner is invited to contact the undersigned at the number provided. Favorable action by the Examiner is earnestly solicited.

AUTHORIZATION

The Commissioner is hereby authorized to charge any additional fees which may be required for consideration of this Amendment to Deposit Account No. **13-4500**, Order No. 4439-4025.

In the event that an extension of time is required, or which may be required in addition to that requested in a petition for an extension of time, the Commissioner is requested to grant a petition for that extension of time which is required to make this response timely and is hereby authorized to charge any fee for such an extension of time or credit any overpayment for an extension of time to Deposit Account No. **13-4500**, Order No. 4439-4025.

Respectfully submitted,
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Dated: November 26, 2007 By: 

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Neurosci Lett. 2004 Jun 17;363(3):243-6.

Preventive effect of antioxidants in MPTP-induced mouse model of Parkinson's disease.

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Oxidative stress to dopaminergic neurons is believed to be one of the causes of neurodegeneration in Parkinson's disease (PD). It was investigated whether N-acetylcysteine (NAC) and l-2-oxothiazolidine-4-carboxylate (OTC) have a preventive effect in an oxidative stress-induced model of PD. We found that NAC and OTC prevent degradation of PARP during auto-oxidized dopamine- or auto-oxidized L-DOPA-induced apoptosis in PC12 cells. In an animal model study, NAC and OTC showed a preventive effect against MPTP-induced loss of tyrosine hydroxylase-positive neurons, and suppressed the nuclear translocation of c-jun N-terminal kinase (JNK), suggesting that NAC and OTC can prevent MPTP-induced apoptosis by suppressing JNK activation. Therefore, these results suggest that NAC and OTC can be used as potential agents to prevent the progression of PD.

PMID: 15182952 [PubMed - indexed for MEDLINE]

Use of autoantigen-knockout mice in developing an active autoimmune disease model for pemphigus

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Received for publication October 21, 1999, and accepted in revised form January 26, 2000.

The development of experimental models of active autoimmune diseases can be difficult due to tolerance of autoantigens, but knockout mice, which fail to acquire tolerance to the defective gene product, provide a useful tool for this purpose. Using knockout mice lacking desmoglein 3 (Dsg3), the target antigen of pemphigus vulgaris (PV), we have generated an active disease model for this autoantibody-mediated disease. *Dsg3*$^{-/-}$ mice, but not *Dsg3*$^{+/-}$ littermates, produced anti-Dsg3 IgG that binds native Dsg3, when immunized with recombinant mouse Dsg3. Splenocytes from the immunized *Dsg3*$^{-/-}$ mice were then adoptively transferred into *Rag-2*$^{-/-}$ immunodeficient mice expressing Dsg3. Anti-Dsg3 IgG was stably produced in the recipient mice for more than 6 months without further boosting. This IgG bound to Dsg3 in vivo and disrupted the cell-cell adhesion of keratinocytes. Consequently, the recipient mice developed erosions in their oral mucous membranes with typical histologic findings of PV. In addition, the recipient mice showed telogen hair loss, as found in *Dsg3*$^{-/-}$ mice. Collectively, the recipient mice developed the phenotype of PV due to the pathogenic anti-Dsg3 IgG. This model will be valuable for developing novel therapeutic strategies. Furthermore, our approach can be applied broadly for the development of various autoimmune disease models.

J. Clin. Invest. **105**:625–631 (2000).

Introduction

Self-tolerance is acquired as a result of clonal deletion or the inactivation of developing lymphocytes that are potentially harmful to the body (1–3). This prevents the immune system from reacting destructively against self components, which can lead to devastating autoimmune diseases. On the other side of the same coin, however, it is very difficult to develop experimental models for autoimmune diseases, which are pivotal for dissecting the mechanisms of tolerance and autoimmunity, as well as for developing novel therapeutic strategies. In this study, we attempted to overcome this difficulty by using autoantigen-knockout mice. In these mice, self-tolerance of the defective gene product is not acquired because lymphocytes are never exposed to the target antigen during development. Adoptive transfer of lymphocytes from autoantigen-knockout mice after immunization with the antigen, into mice expressing the antigen, should generate an autoimmune reaction in the recipient mice, thus providing an active disease model for autoimmune disease. To test this hypothesis, we used a well-defined autoimmune disease against skin and mucous membranes, pemphigus vulgaris (PV).

PV is a life-threatening autoimmune disease of the skin and mucous membranes that is histologically characterized by blister formation due to the loss of cell-cell adhesion of keratinocytes, and immunopathologically by the presence of in vivo bound and circulating IgG directed against the cell surface of keratinocytes in vivo (4). Clinically, patients with PV develop widespread flaccid blisters and painful erosions, which can occur in any stratified squamous epithelium. The target antigen of PV, desmoglein 3 (Dsg3), is a transmembrane desmosomal protein that belongs to the cadherin supergene family of cell-cell adhesion molecules (5–7). Compelling evidence has accumulated for the pathogenicity of IgG autoantibodies against Dsg3 in PV (8–12).

In this study, we developed an active autoimmune disease model of PV using mice that are genetically deficient in the target antigen for PV. We immunized *Dsg3*$^{-/-}$ mice (13) with mouse recombinant Dsg3 (rDsg3), and then adoptively transferred their splenocytes into *Rag-2*$^{-/-}$ immunodeficient mice that express Dsg3. The recipient mice stably produced the pathogenic anti-Dsg3 IgG and exhibited the phenotype of PV. Our approach can be widely applied in developing experimental models of various autoimmune diseases.

Methods

Construction of recombinant mouse Dsg3 and Dsg1 protein. A cDNA encoding the entire extracellular domain of mouse Dsg3 (GenBank U86016) was PCR amplified on

a phage clone containing mouse Dsg3 cDNA as a template (a kind gift from Jouni Uitto, Jefferson Medical College, Philadelphia, Pennsylvania, USA) with the appropriate primers (5′-CCGAGATCTCCTATAAATATGACCTGCCTCTTCCCTAGA-3′ and 5′-CGGGTCGACCCTCCAGGATGACTCCCCATA-3′). In the same way, a cDNA encoding the entire extracellular domain of mouse Dsg1, the autoantigen of pemphigus foliaceus, was PCR amplified on a plasmid clone containing mouse Dsg1 cDNA (a kind gift from Norihisa Matsuyoshi, and John R. Stanley, University of Pennsylvania; and Leena Pulkinen, and Jouni Uitto, Jefferson Medical College) with another pair of primers (5′-CCGAGATCTCCTATAAATATGGACTGGCACTCCTTCAGG-3′ and 5′-CGGCTCGAGGTGAACGTTGTCTCCATAGAG-3′). These cDNAs were subcloned into pEVmod-Dsg3-His vector (14) in place of cDNA for human Dsg3 (pEVmod-mDsg3-His, pEVmod-mDsg1-His). Recombinant baculoproteins, mouse rDsg3 and rDsg1, were prepared as previously described (15, 16).

Mice. *Dsg3*$^{-/-}$ mice were obtained by mating male *Dsg3*$^{-/-}$ mice and female *Dsg3*$^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, Maine, USA) (13). *Dsg3*$^{-/-}$ mice have a mixed genetic background of 129/SV (H-2^{b}) and C57BL/6J (H-2^{b}) (13). *Rag-2*$^{-/-}$ mice that had been backcrossed to B6.SJL-*Ptprc*^{a} mice for 10 generations were obtained from Taconic Farms (Germantown, New York, USA) (17).

ELISA. Circulating anti-Dsg3 IgG was measured by ELISA using mouse rDsg3 as a coated antigen as previously described (14, 18). Each sample was diluted 50-fold and run in duplicate. A single serum sample obtained from a *Dsg3*$^{-/-}$ mouse immunized with mouse rDsg3 was used as a positive control, and serum from a nonimmunized mouse was used as a negative control. ELISA scores were obtained as index values with the following formula: index value = (OD_{450} of sample − OD_{450} of negative control)/(OD_{450} of positive control − OD_{450} of negative control) × 100 (ref. 18). When the OD exceeded 2.0, the serum sample was further diluted and the index value was multiplied by the dilution factor. The ELISA scores against mouse rDsg1 were measured in the same way using rDsg1-coated ELISA plates.

Living keratinocyte staining. A mouse keratinocyte cell line, PAM212 (19), was incubated with mouse serum samples diluted 20-fold with DMEM containing 10% FCS at 37°C in a CO_2 incubator for 30 minutes. After being washed with PBS, the cells were fixed with 100% methanol at −20°C for 20 minutes, and incubated with FITC-conjugated goat anti-mouse antibodies (DAKO A/S, Glostrup, Denmark) at room temperature for 30 minutes. Specimens were examined under an Eclipse E800 fluorescent microscope (Nikon Corp. Tokyo, Japan).

Immunization of mice. Mice were primed by intraperitoneal injection of 5 μg of purified mouse rDsg3 in complete Freund's adjuvant on day 0. They were subsequently boosted with mouse rDsg3 in incomplete Freund's adjuvant twice, and then injected with mouse rDsg3 without adjuvant twice each week. Antibody production was examined by ELISA at the indicated time.

Adoptive transfer of splenocytes. Splenocytes were isolated from *Dsg3*$^{-/-}$ or *Dsg3*$^{+/-}$ mice on day 32 (4 days after boosting with mouse rDsg3 without adjuvant at day 28). Typically, the splenocytes were pooled from 2 immunized *Dsg3*$^{-/-}$ or *Dsg3*$^{+/-}$ mice and then administered to 10 *Rag-2*$^{-/-}$ mice. Intravenous injection into the tail vein of *Rag-2*$^{-/-}$ mice was used to transfer 10^{7} splenocytes in 500 μL PBS per mouse.

ELISPOT assay. PVDF-bottomed 96-well Amicon multititer plates (Millipore Corp., Beverly, Massachusetts, USA) were coated with 30 μg/mL of mouse rDsg3. Mononuclear cells prepared from the peripheral blood, spleen, bone marrow, and lymph nodes of reconstructed *Rag-2*$^{-/-}$ mice were incubated on the plates at 37°C in a CO_2 incubator for 4 hours. IgG bound to the membrane was revealed as spots with alkaline phosphatase–conjugated anti-mouse IgG antibodies (Zymed Laboratories Inc., South San Francisco, California, USA). The number of spots was counted under a dissecting microscope, and the frequency of anti-Dsg3 IgG-producing B cells was defined as the number of spots in 10^{5} mononuclear cells. All experiments were carried out in triplicate.

Results

Dsg3$^{-/-}$ mice, but not wild-type mice, produced anti-Dsg3 IgG capable of binding to the native mouse Dsg3 on living keratinocytes. Initially, we tested various wild-type mice (C57BL/6N, BALB/c, and C3H/HeJ) for their ability to produce anti-Dsg3 IgG upon immunization. However, none of them produced IgG that was able to recognize the native form of mouse Dsg3 (data not shown). We believe this failure was probably due to immunologic tolerance against mouse Dsg3, a self-antigen.

Such tolerance should not be acquired in mice that are genetically deficient in Dsg3. When we immunized *Dsg3*$^{-/-}$ mice with mouse rDsg3, anti-Dsg3 IgG was indeed produced. The ELISA titers against rDsg3 became positive at day 11 in *Dsg3*$^{-/-}$ mice, and their titers continued to increase thereafter (Figure 1a). These sera were able to bind to the cell surfaces of living cultured mouse keratinocytes (Figure 1b, left), indicating that the anti-Dsg3 IgG produced in *Dsg3*$^{-/-}$ mice is capable of binding to the native Dsg3 on living keratinocytes. In contrast, although the ELISA titers of *Dsg3*$^{+/-}$ littermates became positive after repeated immunization, their titers were lower than those of *Dsg3*$^{-/-}$ mice (day 33, $P = 0.0016$). More importantly, the sera from *Dsg3*$^{+/-}$ mice failed to bind to the surface of living keratinocytes (Figure 1b, right). In addition, there was no in vivo deposition of IgG in the stratified squamous epithelia of the immunized *Dsg3*$^{-/-}$ mice (data not shown). Therefore, most of the antibodies developed in *Dsg3*$^{+/-}$ mice may have been raised against minor contaminants of the recombinant protein preparation, tags introduced at the COOH-terminus of rDsg3, or degraded products. These findings indicate

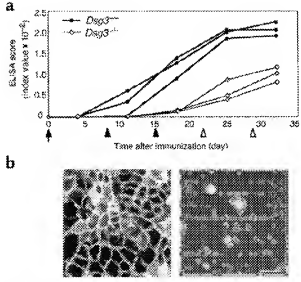

Figure 1
Anti-Dsg3 IgG antibodies that can bind the native Dsg3 in vivo are produced in *Dsg3*$^{-/-}$ mice, but not in *Dsg3*$^{+/-}$ mice. (**a**) *Dsg3*$^{-/-}$ mice and their *Dsg3*$^{+/-}$ littermates were immunized with mouse rDsg3, and the ELISA titers against rDsg3 were measured over time. Mice were primed by intraperitoneal injection of purified mouse rDsg3 in complete Freund's adjuvant on day 0 (arrow). They were subsequently boosted with mouse rDsg3 in incomplete Freund's adjuvant (solid triangles), and then injected with the mouse rDsg3 without adjuvant (open triangles). (**b**) A mouse keratinocyte cell line, PAM212, was incubated with mouse serum samples in culture media in a CO_2 incubator for 30 minutes. After being washed and fixed with methanol, bound mouse IgG was revealed with FITC-conjugated goat anti-mouse IgG antibodies. Sera from immunized *Dsg3*$^{-/-}$ mice (left), but not from their *Dsg3*$^{+/-}$ littermates (right), stained the cell-cell contact sites of cultured keratinocytes. Bar, 50 μm.

that *Dsg3*$^{-/-}$ mice and mice expressing Dsg3 have a clear difference in their ability to produce anti-Dsg3 IgG that can bind to native Dsg3.

Stable production of anti-Dsg3 IgG by lymphocytes of immunized Dsg3$^{-/-}$ *mice in recipient Rag-2*$^{-/-}$ *mice.* Despite the production of anti-Dsg3 IgG, no autoimmune reaction was expected in the immunized *Dsg3*$^{-/-}$ mice because these mice lack the target antigen. To allow the anti-Dsg3 IgG to be exposed to the antigen, we isolated splenocytes from the immunized *Dsg3*$^{-/-}$ mice, or from *Dsg3*$^{+/-}$ mice as controls, and transferred them into *Rag-2*$^{-/-}$ immunodeficient mice (17) that do express Dsg3. *Rag-2*$^{-/-}$ mice have no mature T or B cells, due to their inability to rearrange T-cell receptor or immunoglobulin genes, and thus are unable to produce antibodies or to reject the transferred splenocytes.

Circulating anti-Dsg3 IgG was detected in the sera of recipient *Rag-2*$^{-/-}$ mice as early as day 4 after the transfer of *Dsg3*$^{-/-}$ splenocytes. The level increased rapidly without further boosting by rDsg3, and reached a plateau around day 21 (Figure 2a). The circulating anti-Dsg3 IgG was detected for as long as these mice were assayed, which was more than 6 months, indicating that endogenous Dsg3 in the recipient mice stimulated the transferred Dsg3-specific lymphocytes from the immunized *Dsg3*$^{-/-}$ mice in vivo. Furthermore, the ELISPOT assay also detected anti-Dsg3 IgG-producing B cells in the spleen and lymph nodes of the recipient mice 4 months after the adoptive transfer (Table 1), confirming the persistent ongoing antibody production. No significant reactivity against Dsg1, another desmosomal cadherin targeted in pemphigus foliaceus, was observed in these recipient mice during this period (Figure 2a). In marked contrast, no circulating anti-Dsg3 IgG was detected in *Rag-2*$^{-/-}$ mice given *Dsg3*$^{+/-}$ splenocytes (Figure 2a), although the numbers of CD4$^+$ T cells and CD19$^+$ B cells from *Dsg3*$^{+/-}$ mice were comparable with those from *Dsg3*$^{-/-}$ mice in the recipient mice (data not shown).

Rag-2$^{-/-}$ *mice with immunized Dsg3*$^{-/-}$ *splenocytes develop PV phenotype.* The first symptom we noticed in the recipient *Rag-2*$^{-/-}$ mice that received immunized *Dsg3*$^{-/-}$ splenocytes was weight loss that began between day 7 and day 14 after the adoptive transfer (Figures 2b and 3a, Table 2). In the following days, these mice continued to lose weight, and some of them died. The mice that survived started to gain weight again about day 35. Some of these recipient *Rag-2*$^{-/-}$ mice developed crusted erosions on the skin around the snout (Figure 3b), an area that is normally traumatized by scratching.

In *Rag-2*$^{-/-}$ mice that received immunized *Dsg3*$^{-/-}$ splenocytes, in vivo IgG deposition was found on keratinocyte cell surfaces in stratified squamous epithelium, including the skin (Figure 3c, the skin around the snout), and oral (Figure 3d, hard palate) and esophageal mucous membranes, just as is seen in patients with PV (Figure 3f). In the epidermis where there were multiple layers of keratinocytes, IgG deposition was limited to the lower layers, whereas in the oral and esophageal epithelium, IgG was found

Table 1
Detection of anti-Dsg3 IgG-producing B cells by ELISPOT assays in *Rag-2*$^{-/-}$ mice injected with immunized *Dsg3*$^{-/-}$ splenocytes

Mouse	Transfer	Day[A]	Spleen	Lymph node	Bone marrow	PBMC
#466	–	–	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
#514	+	22	86.5 ± 29.9	13.5 ± 13.6	0.0 ± 0.0	3.8 ± 5.4
#212	+	33	102.1 ± 14.7	47.8 ± 8.8	0.0 ± 0.0	0.0 ± 0.0
#134	+	117	20.8 ± 5.9	16.5 ± 5.9	2.1 ± 2.9	0.0 ± 0.0
#135	+	117	31.3 ± 8.8	27.1 ± 2.9	0.0 ± 0.0	0.0 ± 0.0

Rag-2$^{-/-}$ mice were injected with (+) or not injected with (–) splenocytes of immunized *Dsg3*$^{-/-}$ mice. [A]Number of days after transfer that sacrifice was performed. Number of Anti-Dsg3 IgG-producing B cells is shown per 10^6 mononuclear cells in spleen, lymph nodes, bone marrow, and PBMC.

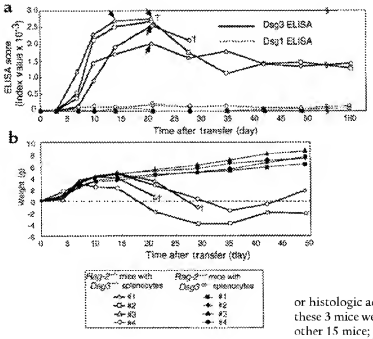

Figure 2
Stable production of anti-Dsg3 IgG in mice given immunized *Dsg3*$^{-/-}$ splenocytes. Splenocytes were isolated from immunized *Dsg3*$^{-/-}$ or *Dsg3*$^{+/-}$ mice and then transferred to *Rag-2*$^{-/-}$ mice by intravenous injection into the tail vein. (**a**) Circulating anti-Dsg3 IgG was detected in the mice that received *Dsg3*$^{-/-}$ splenocytes (open symbols, solid line), but not in the recipients of *Dsg3*$^{+/-}$ splenocytes (solid symbols). The antibody production persisted for more than 6 months. There was no apparent reactivity against Dsg1 (dashed line). Arrows indicate the day when the hair loss phenotype became apparent. (**b**) Weight change in the recipient *Rag-2*$^{-/-}$ mice is shown by plotting weight against time. †indicates the death of a mouse.

throughout the layers of living epithelium. In these mice, no IgG deposition was found in other tissues, including heart, lung, liver, kidney, stomach, and small and large intestines (data not shown). These IgG binding sites correspond to the known tissue distribution of Dsg3 (5, 20, 21).

Histologic examination of the recipient mice revealed an intraepithelial loss of cell-cell adhesion just above the basal layers (i.e., suprabasilar acantholysis), in the buccal mucosa, hard palate (Figure 3g), oropharyngeal areas, and the upper part of the esophagus (Figure 3h). These are typical histologic findings in PV patients (Figure 3j). These oral erosions probably inhibited food intake, resulting in the weight loss. No significant infiltration of inflammatory cells was observed in the early stages of developing blisters (Figure 3, g and h). In contrast, no phenotypic or pathologic changes were observed in *Rag-2*$^{-/-}$ mice that received immunized *Dsg3*$^{+/-}$ splenocytes (Figure 3, e and i). There was no apparent sign of graft-versus-host disease in the skin or intestines in any of these mice given *Dsg3*$^{+/-}$ or *Dsg3*$^{+/+}$ splenocytes (data not shown).

All 18 mice receiving *Dsg3*$^{-/-}$ splenocytes showed positive in vivo IgG deposition on keratinocyte cell surfaces, although 3 of them did not show apparent weight loss or histologic acantholysis (Table 2). The IgG titers in these 3 mice were significantly lower than those in the other 15 mice; peak index values were $(0.7 + 0.4) \times 10^3$ and $(2.4 + 0.9) \times 10^3$, respectively ($P < 0.01$). When mice with lower titers were boosted with rDsg3, the titers rose and the mice developed the PV characteristics (data not shown). Therefore, the appearance of the PV characteristics might generally be correlated with the antibody titer. These results indicate that the *Rag-2*$^{-/-}$ mice given immunized *Dsg3*$^{-/-}$ splenocytes developed clinical, histologic, and immunopathologic characteristics similar to those of PV patients.

Rag-2$^{-/-}$ *mice with immunized Dsg3*$^{-/-}$ *splenocytes develop alopecia.* Around 15–25 days after the adoptive transfer, we also observed patchy hair loss in the *Rag-2*$^{-/-}$ mice that received immunized *Dsg3*$^{-/-}$ splenocytes (see arrows in Figure 2a; Figure 4, a and b), but not in those that received *Dsg3*$^{+/-}$ splenocytes. Typically, the bald areas started as small spots and gradually enlarged peripherally over the next 2–3 weeks. In some mice, new hair grew in the bald areas in a patchy pattern (Figure 4e), whereas in others, the bald areas remained in the same spot for more than 1 month. Some mice had diffuse hair loss with no discrete bald areas. This hair loss also persisted for more than 6 months. Skin biopsy showed intense IgG deposition on the cell surface of keratinocytes surrounding the telogen hair club (Figure 4d). Cleft formation was observed between the cells surrounding the tel-

Table 2
Summary of PV phenotype observed in *Rag-2*$^{-/-}$ recipient mice

Rag-2$^{-/-}$ mice with	Number tested	Positive ELISA[A]	Living cell Staining[B]	Positive DIF[C]	Acantholysis in histology[D]	Weight loss Phenotype[E]	Crusted erosive Lesions	Hair loss Phenotype
Dsg3$^{-/-}$ splenocytes	18	18	18	18	15	15	6	13
Dsg3$^{+/+}$ splenocytes	10	0	0	0	0	0	0	0

Number of mice with positive finding for each category is shown. [A]Circulating IgG was tested with ELISA against mouse rDsg3. [B]Circulating IgG was determined by staining of living keratinocytes (PAM212). [C]In vivo IgG deposition on keratinocyte cell surfaces was determined by direct immunofluorescence staining of oropharyngeal mucous membranes. [D]Intraepithelial blister formation (i.e. suprabasilar acantholysis) was determined by histologic examination of the oropharyngeal areas. [E]Weight loss greater than 2 g within a week was considered significant.

ogen club and the basal layer of the outer root sheath epithelium (Figure 4e). The bald skin contained empty, dilated telogen hair follicles, consistent with the loss of the telogen hair (Figure 4f). These clinical and histologic findings of telogen hair loss are virtually identical to those observed in *Dsg3*$^{-/-}$ mice, as are the findings of erosions on the oral mucous membrane and the skin (13, 22), confirming the specificity and pathogenicity of the anti-Dsg3 IgG produced in these mice.

Discussion

In this study, we adopted a unique approach to develop an active disease mouse model of pemphigus, by assuming that self-tolerance against Dsg3 is lost in *Dsg3*$^{-/-}$ mice. Immunization with mouse rDsg3 was indeed successful in generating anti-Dsg3 IgG capable of binding native mouse Dsg3 on living keratinocytes in *Dsg3*$^{-/-}$ mice, but not in *Dsg3*$^{+/+}$ littermates. When splenocytes containing activated lymphocytes specific for mouse Dsg3 were adoptively transferred to *Rag-2*$^{-/-}$ immunodeficient mice that expressed Dsg3, the Dsg3-specific lymphocytes encountered the endogenous Dsg3, resulting in stable production of anti-Dsg3 IgG antibody for more than 6 months. The adoptive transfer of purified T cells and B cells from *Dsg3*$^{-/-}$ mice was sufficient to produce such anti-Dsg3 IgG (M. Amagai et al., unpublished data), excluding the possible involvement of other types of cells — including antigen-presenting cells from *Dsg3*$^{-/-}$ mice — in this autoantibody production. In contrast, the endogenous Dsg3 did not stimulate lymphocytes from immunized *Dsg3*$^{-/-}$ mice; no anti-Dsg3 IgG was produced in the mice receiving those lymphocytes. The persistent ongoing production of IgG against Dsg3 in the *Rag-2*$^{-/-}$ mice given *Dsg3*$^{-/-}$ splenocytes up to 4 months after the transfer was confirmed by the ELISPOT assay. Furthermore, the anti-Dsg3 IgG was pathogenic, and the recipient *Rag-2*$^{-/-}$ mice developed symptoms of PV, including erosions in the mucous membranes, which inhibited food intake with resultant weight loss, and scaly crusted erosions on traumatized skin, which resembles Nikolsky's sign (gentle rubbing of normal-appearing skin induces blisters). Consequently, this new approach has provided us with a novel active disease animal model of PV.

Clinically, PV is divided into a mucosal-dominant type and a mucocutaneous type. In mucosal-dominant PV, oral erosions predominate, with limited skin involvement. In mucocutaneous PV, there are extensive skin lesions in addition to oral involvement. Recently, it was demonstrated that this clinical difference is defined by the anti-Dsg autoantibody profile (14, 23–25). Patients with mucosal-dominant PV have anti-Dsg3 IgG alone, whereas patients with mucocutaneous PV have anti-Dsg1 IgG in addition to anti-Dsg3 IgG. In the epidermis, Dsg1 is coexpressed with Dsg3. Therefore, anti-Dsg3 IgG alone is not able to cause skin blisters efficiently. On the other hand, in the mucous membrane Dsg3 is the dominant Dsg isotype, and anti-Dsg3 IgG alone is sufficient to cause oral erosions. In our model, *Dsg3*$^{-/-}$ mice did not develop anti-Dsg1 IgG that was able to access the native Dsg1: no in vivo IgG deposition was observed in the immunized *Dsg3*$^{-/-}$ mice that do express Dsg1, even after repeated immunization (data not shown). Furthermore, the *Rag-2*$^{-/-}$ mice

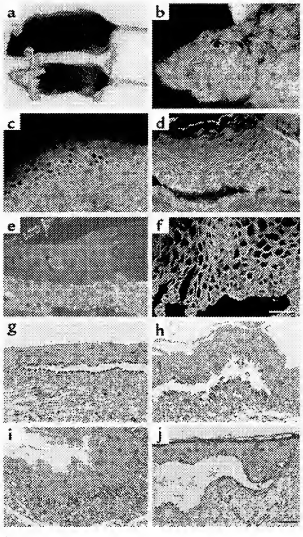

Figure 3

Rag-2$^{-/-}$ mice injected with immunized *Dsg3*$^{-/-}$ splenocytes develop the PV phenotype. (**a**) Mice receiving *Dsg3*$^{-/-}$ splenocytes (bottom) were significantly smaller than mice given *Dsg3*$^{+/+}$ splenocytes (top) 25–35 days after the adoptive transfer. (**b**) Some mice injected with *Dsg3*$^{-/-}$ splenocytes developed crusted erosions around the snout and cheeks, where mice normally scratch. (**c–j**) Histologic and immunopathologic examination of *Rag-2*$^{-/-}$ recipient mice and patients with PV. In vivo IgG deposition on keratinocyte cell surfaces was observed in the skin (**c**, the skin around the snout) and mucous membranes (**d**, hard palate) of *Rag-2*$^{-/-}$ mice given *Dsg3*$^{-/-}$ splenocytes, just as is found in patients with PV (**f**, esophagus biopsy specimen). In contrast, there was no in vivo IgG deposition in the mice given *Dsg3*$^{+/+}$ splenocytes (**e**, hard palate). The mice that received *Dsg3*$^{-/-}$ splenocytes developed intraepithelial blisters just above the basal layers in mucosal epithelium (**g**, hard palate; **h**, upper esophagus), which is a typical histologic finding in PV patients (**j**, skin). There was no apparent sign of acantholysis in the mice with *Dsg3*$^{+/+}$ splenocytes (**i**, upper esophagus). Bars = 50 μm.

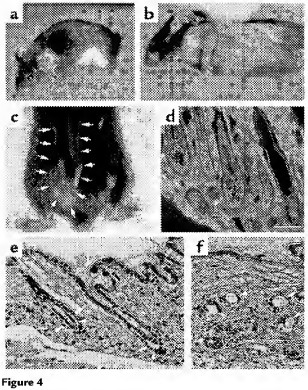

Figure 4
Hair loss phenotype of *Rag-2*$^{-/-}$ mice given splenocytes from immunized *Dsg3*$^{-/-}$ mice. (**a**, **b**) The *Rag-2*$^{-/-}$ mice given *Dsg3*$^{-/-}$ splenocytes showed patchy hair loss, which first became apparent around day 15–25. (**c**) New hair growing in a bald area in a patchy pattern (arrows). (**d**) Intense in vivo IgG deposition was noticed on the cell surface of keratinocytes surrounding the telogen hair club. (**e**, **f**) Cleft formation between the cells surrounding the telogen club and the basal layer of the outer root sheath epithelium (**e**, arrows) and empty, dilated telogen hair follicles (**f**, arrows). Bars = 100 μm.

receiving *Dsg3*$^{-/-}$ splenocytes did not develop any apparent anti-Dsg1 IgG with time (Figure 2a). This finding is consistent with our observation that the phenotypes of recipient mice were virtually identical to those of *Dsg3*$^{-/-}$ mice. The IgG produced in these experiments was essentially specific for Dsg3, indicating that our PV model represents the mucosal-dominant type of PV.

Among the PV phenotypes that the recipient mice developed, in vivo IgG deposition on keratinocyte cell surfaces was found in all 18 mice injected with *Dsg3*$^{-/-}$ splenocytes (Table 2). This in vivo antibody binding to the target antigen is the first and essential step leading to the development of the PV phenotype. Acantholysis in the oral mucosa with resultant weight loss was observed in 15 of the 18 mice; the 3 remaining mice had lower titers of anti-Dsg3 IgG. Telogen hair loss was observed less frequently than was acantholysis or weight loss (Table 2). In mice, hair follicles in any particular area cycle in a synchronous fashion and enter the same stage at approximately the same time (26). This tendency becomes less evident as mice age. In addition, for a certain period mice have a normal coat, because hair regrows when the hair cycle enters the anagen stage (22). Therefore, the hair loss phenotype might have been missed because some mice died or were sacrificed before the hair loss became apparent.

Previously, an in vivo experimental model of PV was developed by reconstituting severe combined immunodeficiency (SCID) mice with PBMC from patients with PV (27). In that model, lymphocytes from the patients produced a low titer of circulating anti-human Dsg3 IgG_1 but the spontaneous intraepidermal blisters associated with human IgG deposits were seen only rarely in the mouse skin. After grafting human skin on the SCID mice, PV-like blisters were observed in the grafted skin, although it remains to be determined whether inflammatory reactions due to possible histoincompatibility of human PBMC and skin contributed to the blister development in that model. Therefore, the mouse model developed in our study is the first solid active disease model of PV.

A major hurdle in developing animal models of autoimmune diseases has been overcoming self-tolerance. To create our model of disease, we circumvented this problem by immunizing autoantigen-knockout mice with the autoantigen, then transferring their splenocytes to *Rag-2*$^{-/-}$ mice that expressed the autoantigen. Although this model does not address the usual triggers of autoimmune diseases, it does provide a means to investigate the roles of T and B lymphocytes in perpetuating autoantibody production in the autoimmune response. In addition, this active animal model should be beneficial for evaluating various therapeutic strategies that could modulate the autoimmune response. Finally, because it is very easy to evaluate disease activity in this model through weight loss and hair loss, it can be used for efficient screening of various therapeutic interventions.

Our approach is widely applicable to various antibody-mediated and T cell–mediated autoimmune diseases (excluding cases where the relevant autoantigen-knockout mice are embryonic lethal or show gross abnormalities in their immune systems). Furthermore, this approach provides another dimension in the use of knockout mice for study of the function of target molecules in vivo.

Acknowledgments

We thank John R. Stanley for reviewing the manuscript and for insightful discussion, and Tasuku Honjo for valuable discussion. We also thank John R. Stanley and Leonard D. Shultz for breeding pairs of *Dsg3*$^{-/-}$ mice, Jouni Uitto for providing mouse Dsg3 cDNA, and Norihisa Matsuyoshi, John R. Stanley, Leena Pulkinen, and Jouni Uitto for providing mouse Dsg1 cDNA. Thanks also go to Akihiro Umezawa for preparing pathologic slides, Yoshiko Fujii for cell culture and for preparing recombinant baculoproteins, Minae Suzuki for immunofluorescence staining, and Ayumi Sakurai for animal care. This work was supported by Health Sciences Research Grants for Research on Specific Diseases from the Ministry of Health and Welfare of Japan

(to M. Amagai), a Grant-in-Aid of Scientific Research (to M. Amagai, S. Koyasu, and T. Nishikawa), a Grant-in-Aid of International Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (to T. Nishikawa), Keio Gijuku Academic Development Funds (to M. Amagai), and a grant from the Japan Society for the Promotion of Science (to S. Koyasu, JSPS-RFTF97L00701).

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